Pestic. Sci. 1998, **53**, 201–208

Proinsecticides Effective against Insecticide-Resistant Peach-Potato Aphid (*Myzus persicae* (Sulzer))

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(Received 15 August 1997; revised version received 21 January 1998; accepted 4 February 1998)

Abstract: A range of potential proinsecticides was synthesised and tested against insecticide-susceptible and -resistant clones of *Myzus persicae* (Sulzer). They were all esters of compounds known to be toxic or pharmacologically active, and were designed to have increased lipophilicity and to be subject to more rapid activation by hydrolysis in resistant than in susceptible aphids due to the increased amount of esterase present in the resistant clones. The most potent toxins were esters of monofluoroacetic acid. When applied topically, the toxicity of these esters to *M. persicae* was directly proportional to the esterase content of the aphids. Such compounds would not be suitable as commercial insecticides, but the results serve to illustrate the potential benefits of exploiting a resistance mechanism against one class of compounds to render another class more toxic, i.e. to design compounds that show negative cross-resistance. © 1998 SCI

Pestic. Sci., **53**, 201–208 (1998)

Key words: *Myzus persicae*; insecticide resistance; esterase; proinsecticides; negative cross-resistance; aphids

1 INTRODUCTION

The peach-potato aphid *Myzus persicae* (Sulzer), a major pest on a variety of crops, has developed resistance worldwide to organophosphorus, carbamate and pyrethroid insecticides through the increased production of a carboxylesterase, E4, or its closely related variant FE4. These enzymes inactivate insecticides by sequestration and ester hydrolysis.[1] In the most highly resistant aphids there is approximately 60 times as much esterase activity as in susceptible aphids, accounting for 1–2% of total body protein. Molecular genetic studies have shown that this increase in esterase production is primarily due to gene amplification, i.e. the presence of multiple copies of the esterase gene in resistant aphids.[2]

Increased esterase production is associated with resistance to insecticides in many species. This has been shown primarily by using 1-naphthyl acetate as a chromogenic substrate both in homogeneous solution assays and for staining electrophoresis gels. Such differences in esterase levels between susceptible and resistant phenotypes offer the possibility of using proinsecticidal esters for the preferential control of the resistant variants. Negative cross-resistance (i.e. a resistance mechanism to one class of insecticides conferring increased sensitivity to another class) is a major, so far unachieved, goal for incorporation into insecticide resistance-management strategies. The best-documented instance of negative cross-resistance is in the green rice leafhopper *Nephotettix cincticeps* (Uhl), in which the target enzyme, acetylcholinesterase, has become insensitive to the widely used *N*-methylcarbamates but, by the same mutation, has been rendered hypersensitive to *N*-propylcarbamate insecticides.[3]

Many commercial insecticides are applied as proinsecticides. For example, many organophosphorus compounds are applied as phosphorothionates, requiring oxidative activation to the phosphate to become potent

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Contract/grant sponsor: Biotechnology and Biological Sciences Research Council, UK.

cholinesterase inhibitors. Likewise, a number of carbamate insecticides have been derivatised (e.g. as sulfenyl compounds) to render them less toxic to mammals yet be readily activated by the target insect.[4] There are other non-commercialised examples. Kochansky *et al.*[5] reported the use of delayed-action fluoroalkyl esters in the control of fire-ants, Prestwich *et al.*[6–8] investigated the insect-specific metabolism of fluorinated lipids in the termite *Regiculitermis flavipes* (Kollar) and the tobacco horn-worm *Manduca sexta* (Joh), and Palmer *et al.*[9,10] studied the selective toxicity to houseflies of substituted bicyclooctane GABA antagonists. However, these examples did not aim to exploit the biochemical mechanisms of resistance to other insecticides.

In the present study we have used ester proinsecticides to exploit a resistance mechanism based on quantitative changes in enzyme expression rather than structural changes in the target protein as in the case of the green rice leafhopper.[3]

2 MATERIALS AND METHODS

2.1 Aphid clones

Apterous adult virginoparae of three *M. persicae* clones were used throughout. Clonal nomenclatures, US1L (susceptible, S), 794J (highly resistant R_3 overproducing esterase E4) and 800F (R_3 overproducing esterase FE4), and origins are given by Sawicki *et al.*[11] and Devonshire *et al.*[12] In one experiment, aphids of intermediate resistance levels (resulting from intermediate esterase content) were used: 405D (R_1 expressing FE4) and T1V (R_2 expressing E4). Broad resistance to established insecticides increases through the series S, R_1, R_2 to R_3.

All aphid clonal lines were maintained at 21($\pm$1·4)°C under a 16/8 h light/dark cycle on Chinese cabbage (*Brassica chinensis* Juslen cv. Tip Top (Brassicaceae)) either on intact plants or on excised leaves in small box-cages.[13] Clonal integrity was checked at regular intervals by staining for esterases after non-denaturing polyacrylamide gel electrophoresis and/or immunoassay.[14]

2.2 Test compounds

Fluoroacetamide was obtained from Sigma.

2.2.1 Compounds in initial screen (Table 1)

In view of the well-established activity of the *M. persicae* esterases in hydrolysing naphthyl acetate and butyrate, 1-naphthol was chosen primarily as the esteratic alcohol. The lead compounds for testing as 1-naphthyl esters were chosen from the literature on insect and mammalian pharmacology and neurochemistry.[15–17]

If any reactive groups were present on an acid in addition to the intended carboxyl group, they were protected before esterification by reaction with benzylchloroformate (-NH groups) or *tert*-butyldimethylsilyl chloride (-OH groups) prior to acid chloride formation. The chosen acids (100–400 mg), protected as necessary, were converted to their acid chlorides by reaction with 1·1 equivalents of thionyl chloride in dichloromethane + pyridine (50 + 1 by volume). To each acid chloride, 1·1–2·2 equivalents of 1-naphthol were added and the mixture stirred overnight at room temperature. Any protecting groups were removed and the products dried over anhydrous magnesium sulfate, then purified by flash column chromatography or distillation.

Aziridinylethyl-*p*-nitrobenzoate was prepared by the slow addition of 0·99 equivalents (666 mg) of *p*-nitrobenzoyl chloride to a 10 ml litre^{-1} solution of 2-aziridinylethanol in dichloromethane. The product was dried over anhydrous magnesium sulfate and evaporated to a yellow oil.

2.2.2 Fluoroacetyl esters (Table 2a)

A slight molar excess (1·1 equivalents) of fluoroacetyl chloride, prepared as above from monofluoroacetic acid (Sigma), was added to a solution of 5–10 mmol of the required alcohol in diethyl ether + pyridine (50 + 1 by volume) at 0°C under nitrogen. The reaction was stirred at room temperature overnight, then poured into water and the mixture extracted into ether. The ethereal extract was washed with base, acid and finally water before being dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The residue was purified by flash column chromatography or distillation.

2.2.3 2-Fluoroethylcyclobutanecarboxylate (Table 2b)

A mixture of 2-fluoroethanol (2·0 mmol), cyclobutane carboxylic acid (2·0 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (4·0 mmol) and *N*,*N*-dimethylaminopyridine (0·2 mmol) in dichloromethane was stirred overnight at room temperature and then applied directly to a flash chromatography column. 2-Fluoroethylcyclobutanecarboxylate was recovered as a colourless oil.

The structures of the synthesised compounds were confirmed by 400 MHz [^{1}H] and [^{13}C] NMR spectroscopy using a Jeol JNM-GX400 FT-NMR spectrometer.

2.3 Bioassay

A topical application bioassay was used based on that described by Needham and Devonshire.[18] Discs (38 mm diameter) cut from Chinese cabbage leaves were placed with their adaxial surface in contact with 12 g litre^{-1} agar in small polyethylene pots coated internally with Fluon to prevent aphid escape. Ten adult apterous *M. persicae* were placed on each leaf disc (three discs per treatment for each clone) and allowed to settle overnight. A drop (0·25 ml) of acetone alone (as control) or containing test material (10–10^4 mg litre^{-1}) was placed on the back of each aphid using an all-glass 1-ml

TABLE 1
Esters Screened for Proinsecticidal Activity

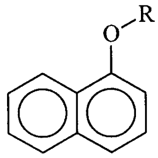
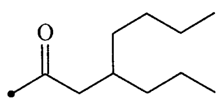
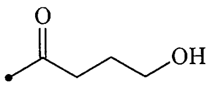
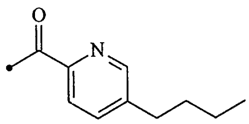
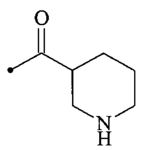
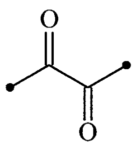
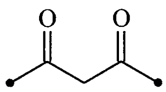
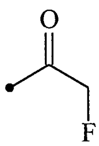
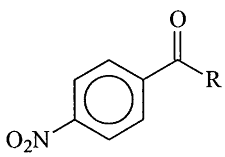
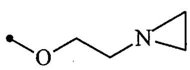
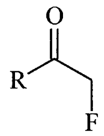
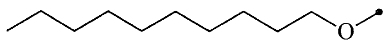
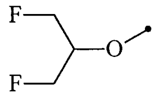
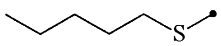
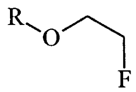
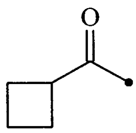
Compound	*R*	*Toxophore*	*Action*
a 1-naphthyl esters			
1		Valproic acid	Anticonvulsant in mammals, acting at multiple sites including GABA receptors
2		γ-Hydroxybutyric acid	GABA antagonist
3		Fusaric acid	Dopamine-β-hydroxylase inhibitor
4		Nipecotic acid	GABA transport inhibitor
5		Oxalic acid	Calcium chelator
6		Malonic acid	Competetive inhibitor of succinate dehydrogenase
7		Fluoroacetic acid	Lethal synthesis of 2-*erythro*-fluorocitric acid inhibits aconitate dehydratase and the mitochondrial citrate transporter
b Para-nitrobenzoyl ester			
8		Aziridinyl ethanol	Inhibitor of cholinergic neurotransmission

TABLE 2
Fluoro Esters Tested as Proinsecticides

Compound	*R*
a Fluoroacetyl esters	
9	
10	
11	
12	NH_2
b Fluoroethyl ester	
13	

syringe depressed by an Arnold manually-operated micrometer-driven applicator (Burkard, UK).

In experiments to determine the rate of intoxication by test compounds, aphids were inspected under a binocular microscope at intervals from 1 h up to 24 h and classified as 'not affected', 'affected' or 'dead'. Those classified as 'affected' had lost their righting reflex, were supine and twitching or immobile but twitched when touched. Since most of those 'affected' had not recovered by the end of the experiments the numbers of affected and dead aphids were pooled for analysis. Abbott's formula[19] was used to account for the effect of the solvent alone. Only some of the data were suitable for estimation of ET_{50} (time for 50% to be affected) values,[20] so the findings are presented as progress curves, which demonstrate the important trends very clearly.

Bioassays were also carried out to determine end-point LC_{50} values for the compounds that appeared to be most selective for the resistant clones, in terms of rate of intoxication. Susceptible (US1L) and resistant (800F) aphids were treated with a range of concentrations (from 0 to 3000 mg litre^{-1} depending on the effective range in the rate experiments) of some of the better compounds showing rapid effects and differential toxicity between strains. Aphid mortality was determined 48 h after treatment. The data were analysed using the POLO statistical analysis program (LeOra Software, 1987) to obtain the LC_{50}, 95% confidence limits and slope of the regression line.

Table 1 gives details of the compounds tested initially on 794J (R_3) aphids at 1000 mg litre^{-1} as described. Once fluoroacetate had been identified as the most promising toxin, a series of esters of this acid with aliphatic alcohols was evaluated (Table 2).

3 RESULTS AND DISCUSSION

3.1 Initial screen

Of the compounds shown in Table 1, only the 1-naphthyl esters of valproic acid (**1**) and monofluoroacetic acid (**7**) caused any toxic effects at 1000 mg litre^{-1}. However, subsequent tests with the valproate ester did not give consistent results and experiments with this compound were discontinued. Aziridinylethyl *p*-nitrobenzoate (**8**) was found to have poor solubility in organic solvents but the reasons for the lack of toxicity of the other compounds in the screen, which were all readily soluble in acetone, were not investigated. The intrinsic potency of the potential intoxicants in aphids is

unknown but pharmacokinetic factors will also have influenced the results.

The effectiveness of the fluoroacetate ester was not unexpected. Fluoroacetic acid is a well-known respiratory poison, acting through the 'lethal synthesis' of 2-fluorocitric acid, a 'suicide substrate' for the Krebs cycle enzyme aconitate hydratase and an inhibitor of the mitochondrial citrate transporter.[21]

1-Naphthylfluoroacetate was the only compound from the initial screen to be studied further.

3.2 Follow-up study using 1-naphthylfluoroacetate (7)

Time-courses for compound **7** and subsequent compounds are presented (Figs 1–5) using the mean percentage of intoxicated insects from the three replicates. Preliminary experiments showed that topical application of 0·25 ml of 1600 mg litre^{-1} **7** to the susceptible and most resistant (R_3) clones affected 90–100% of the aphids within 2 h, with indications that the resistant aphids responded faster. Application of 200 mg litre^{-1} had little effect on either clone. An intermediate dose was therefore chosen to determine if the greater effect on the resistant aphids could be accentuated. When applied at 600 mg litre^{-1}, **7** was markedly more effective against the resistant clones than the susceptible one. The R_1 clone showed a response intermediate between the susceptible (S) and highly resistant (R_2 and R_3)

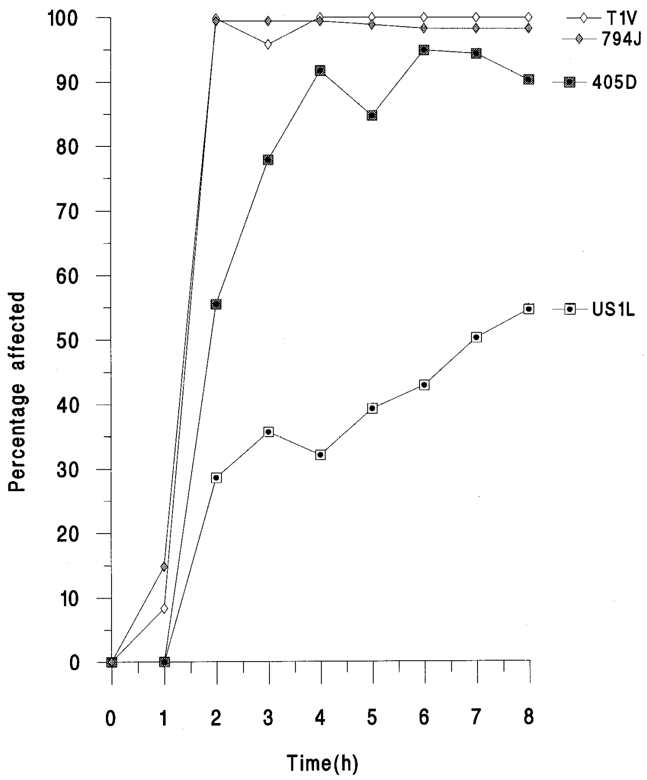


Fig. 1. Effect of topical application of 600 mg litre^{-1} 1-naphthyl fluoroacetate (**7**) on *Myzus persicae* clones classified into different resistance groups US1L (S), 405D (R_1), T1V (R_2) 794J (R_3), according to their esterase content.

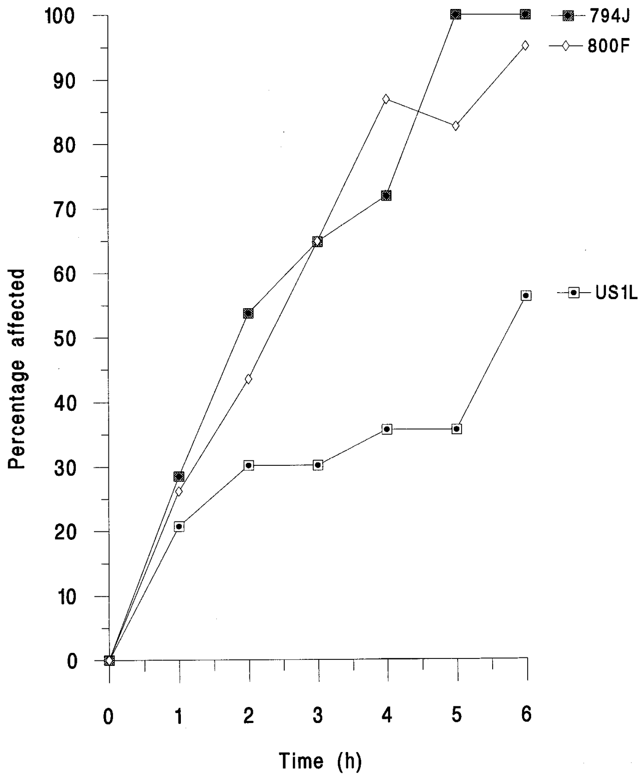


Fig. 2. Effect of topical application of 100 mg litre^{-1} *n*-dodecyl fluoroacetate (**9**) on *Myzus persicae* clones US1L (S), 794J (R_3 overproducing esterase E4) and 800F (R_3 overproducing esterase FE4).

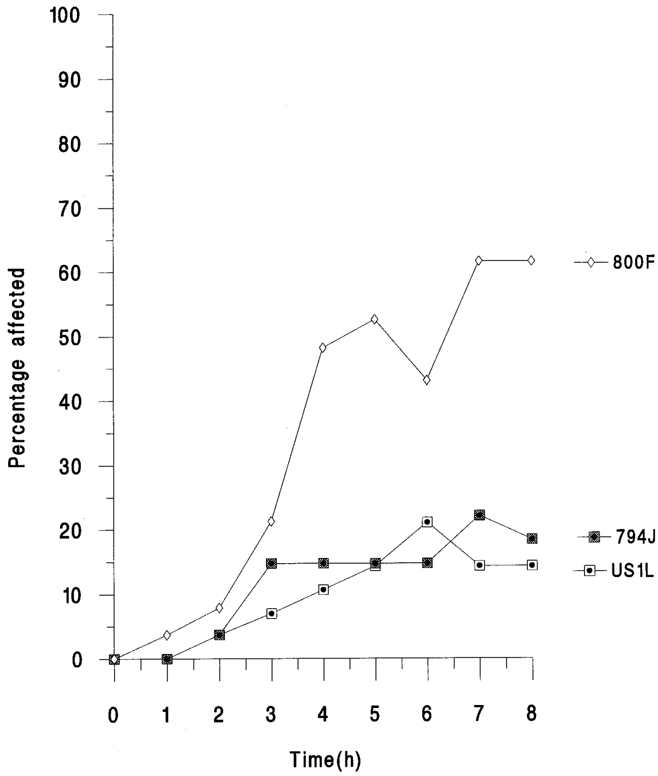


Fig. 3. Effect of topical application of 500 mg litre^{-1} 1,3-difluoroisopropyl fluoroacetate (**10**) on *Myzus persicae* clones US1L (S), 794J (R_3 overproducing esterase E4) and 800F (R3 overproducing esterase FE4).

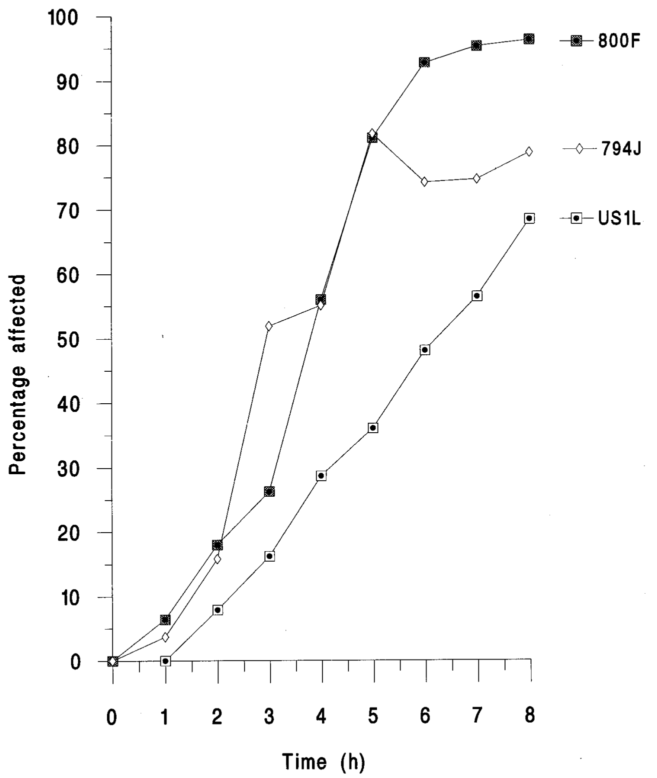


Fig. 4. Effect of topical application of 1000 mg litre^{-1} fluoroacetamide (**12**) on *Myzus persicae* clones US1L (S), 794J (R_3 overproducing esterase E4) and 800F (R_3 overproducing esterase FE4).

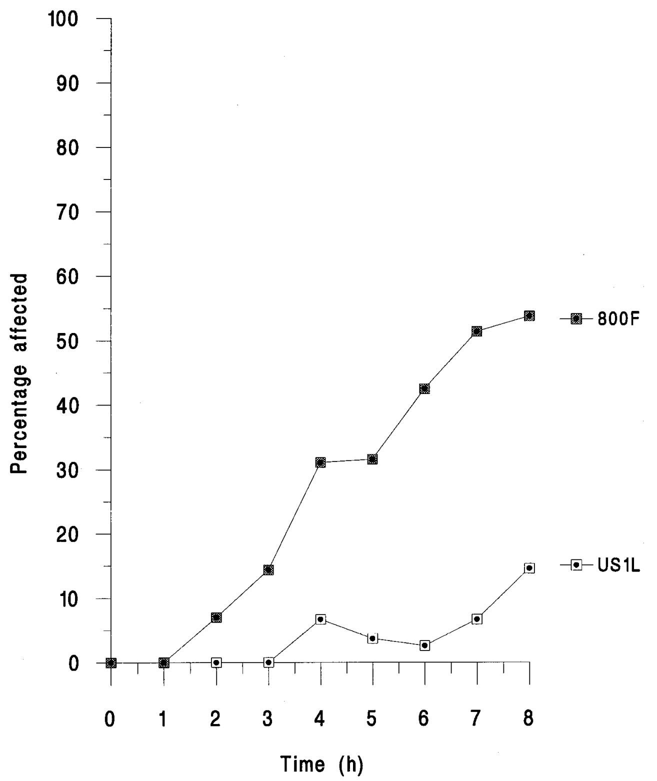


Fig. 5. Effect of topical application of 2000 mg litre^{-1} 2-fluoroethyl cyclobutanecarboxylate (**13**) on *Myzus persicae* clones US1L (S) and 800F (R_3 overproducing esterase FE4).

clones, the latter both being affected to the same extent (Fig. 1). These results suggest that, above a certain level of esterase expression, the rate of toxin production exceeds detoxification.

3.3 Effect of the alcohol moiety on proinsecticidal activity

To investigate whether changing the structure of the substrate might lead to compounds with increased potency and differential activity between high- and low-esterase clones, the alcohol moiety of the proinsecticide was varied. These compounds (Table 2a) were bioassayed using one S and two R_3 clones, one overproducing E4 and the other FE4.

Of the fluoroacetate esters, the most effective were *n*-dodecylfluoroacetate (**9**) and 1,3-difluoroisopropylfluoroacetate (**10**). Treatment with **9** at 100 mg litre^{-1} affected both of the resistant clones more than the susceptible (Fig. 2). Treatment with **10** at 500 mg litre^{-1} affected clone 800F more than 794J or the susceptible aphids (Fig. 3).

It is not clear whether the 1,3-difluoroisopropanol moiety contributes to the observed effects. This alcohol has been proposed as a substitute for fluoroacetate in the control of mammalian pests in Australia.[22] The final toxin is thought to be fluorocitrate but the mammalian oral LD_{50} is about 100 times that of fluoroacetate. This alcohol was therefore chosen in an attempt to increase the aphicidal activity of the proinsecticide by creating a compound with two potentially toxic hydrolysis products. However, subsequent experiments using 1,3-difluoroisopropyliodoacetate and 1,3-difluoroisopropyl-*n*-butyrate showed no significant toxicity in any of the three clones (data not shown), suggesting that the alcohol moiety did not contribute to the aphicidal activity of the fluoroacetate ester.

Pentylthiofluoroacetate (**11**) (100 mg litre^{-1}) had no greater effect on 800F and 794J than on US1L up to 8 h after treatment. At 1000 mg litre^{-1} this thioester appeared to affect the resistant clones more rapidly than the susceptible clone, but by 3 h all three clones were similarly affected. The response to this compound follows the general trend seen with the true esters, but the greater lability of the thioester bond may mask the observation of any negative cross-resistance.

There was some use of fluoroacetamide (**12**) as an aphicide in the 1950s[23] but it was displaced by organophosphorus compounds. Since its activity is dependent on hydrolytic activation to fluoroacetate, it was evaluated in the present study against susceptible and resistant *M. persicae*. At 100 mg litre^{-1} both resistant clones were slightly affected after 8 h, while the susceptible aphids were unaffected. All three clones were affected by 1000 mg litre^{-1}, the resistant ones again significantly more than the susceptible one (Fig. 4).

In-vitro structure–activity experiments using 1-

naphthol esterified with a range of carboxylic acids have suggested that 1-naphthyl cyclobutanecarboxylate is a good substrate for E4 and FE4 (unpublished data). In order to exploit this observation, cyclobutane carboxylic acid was esterified with 2-fluoroethanol. Upon hydrolysis and oxidation with alcohol dehydrogenase and aldehyde dehydrogenase, this ester again yields fluoroacetate and thus fluorocitrate as the final toxin. Therefore 2-fluoroethyl cyclobutanecarboxylate (**13**) (Table 2b) should behave as a proinsecticide like the fluoroacetyl esters, although perhaps more delayed in action because of the increased number of metabolic steps involved.

As expected, **13** affected the resistant clone 800F more than the susceptible clone US1L. At 2000 mg litre^{-1} (Fig. 5) both clones were affected, but the effect on US1L was less and its onset more delayed than that on 800F. Compared with the fluoroacetyl esters, the onset of symptoms was delayed by up to 3 h.

Rapid expression of toxicity can be important in preventing virus transmission. However, for negative cross-resistance to influence selection pressure as part of a resistance management strategy, the differential effects observed so far must also be expressed in terms of end-point toxicity, rather than just knock-down and recovery. Bioassays were therefore done to determine end-point (48 h) toxicity.

3.4 LC_{50} experiments

Compounds **7**, **10** and **13** were lethal to the resistant clone at a concentration significantly lower than that which killed the susceptible clone (Table 3). LC_{50} ratios, US1L/800F, for the three compounds were 2·7, 2·8 and 1·9, respectively.

The lack of discrimination in LC_{50} by **12** correlates with the progress-curve data for this compound, suggesting that fluoroacetamide is not a good substrate for FE4. Hydrolysis of fluoroacetamide in *M. persicae* may therefore be catalysed by an enzyme or enzymes other than the elevated esterases responsible for insecticide resistance.

The LC_{50} data for **9** show no difference between the resistant and susceptible clones, whereas there is a clear difference in the time-course shown in Fig. 2. This implies that the toxicity of this compound depends upon its hydrolysis, some of which is non-enzymic, and in the long term this negates any differential effect of the high esterase content of the resistant insects. Both sets of data, however, show that **9** is toxic at a lower concentration than most of the other compounds tested.

4 CONCLUSION

These data demonstrate the potential of using proinsecticides to control highly resistant *M. persicae* preferentially. The susceptible clone was consistently affected less by most of the treatments tested than were the resistant clones, especially evident in the rate of action, with the FE4-expressing clone 800F usually being the most sensitive. Whether this difference reflects specificity differences between E4 and FE4 or in other pharmacokinetic factors is unknown. However, we have found FE4 to have a larger k_{cat} than E4 for some insecticidal esters and the model substrate 1-naphthyl acetate, which may extend to the ester proinsecticides studied here. Both forms of esterase were active against **7** as judged by the staining of non-denaturing PAGE gels using this substrate (unpublished data). The doses used in the bioassay (100 mg litre^{-1} is equivalent to 25 ng per aphid) are comparable to those of organophosphorus compounds required to kill susceptible aphids and, as a consequence of negative cross-resistance, considerably less than are needed to kill organophosphorus-resistant aphids. However, for such compounds to have wider application, evaluation against a range of species that depend on a high esterase activity for resistance would be needed.

While recognising that the fluoroacetate esters are unlikely to be commercialised because they are probably very toxic to mammals, the work demonstrates the validity of an approach that exploits a resistance-conferring enzyme to render another class of insecticides more toxic. Whilst the concept of negative cross-

TABLE 3
48-h LC_{50} Values for a Range of Fluorinated Compounds Applied Topically to Susceptible and R_3 Clones of *Myzus persicae*

	Susceptible (US1L)			*Resistant, R_3 (800F)*		
Compound	*LC_{50} (g litre^{-1})*	*Limits*	*Slope*	*LC_{50} (mg litre^{-1})*	*Limits*	*Slope*
7	440	350–520	5·9 (±1·5)	160	129–200	3·5 (±0·7)
9[a]	140	110–170	2·0 (±0·2)	130	110–150	5·5 (±1·1)
10[a]	790	660–920	3·3 (±0·6)	280	220–340	2·7 (±0·4)
12	140	90–200	3·3 (±0·7)	170	70–250	3·6 (±1·0)
13[a]	890	580–1260	1·8 (±0·2)	460	270–570	4·0 (±1·1)

[a] Cumulative data from two experiments.

resistance to insecticides is compelling, there are very few known examples and none has been widely exploited commercially. The example presented here is the first to take advantage of a metabolic resistance mechanism.

ACKNOWLEDGEMENTS

The authors thank Mr D. Beddie for the first synthesis of naphthyl fluoroacetate esters, Mr G. J. S. Ross for the statistical treatment of the data and Mrs J. Moir for typing this manuscript. D.H., A.M.H. and R.D.T. were supported by ROPA (Realising Our Potential Award) grants from the Biotechnology and Biological Sciences Research Council of the United Kingdom, which also provides grant-aided support to IACR.

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